Anaerobic Oxidation of Arsenite Linked to Chlorate Reduction[∇][†]

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Microorganisms play a significant role in the speciation and mobility of arsenic in the environment. In this study, the oxidation of arsenite [As(III)] to arsenate [As(V)] linked to chlorate (ClO_3^-) reduction was shown to be catalyzed by sludge samples, enrichment cultures (ECs), and pure cultures incubated under anaerobic conditions. No activity was observed in treatments lacking inoculum or with heat-killed sludge, or in controls lacking ClO_3^-. The As(III) oxidation was linked to the complete reduction of ClO_3^- to Cl^-, and the molar ratio of As(V) formed to ClO_3^- consumed approached the theoretical value of 3:1 assuming the e^- equivalents from As(III) were used to completely reduce ClO_3^-. In keeping with O_2 as a putative intermediate of ClO_3^- reduction, the ECs could also oxidize As(III) to As(V) with O_2 at low concentrations. Low levels of organic carbon were essential in heterotrophic ECs but not in autotrophic ECs. 16S rRNA gene clone libraries indicated that the ECs were dominated by clones of *Rhodocyclaceae* (including *Dechloromonas*, *Azospira*, and *Azonexus* phylotypes) and *Stenotrophomonas* under autotrophic conditions. Additional phylotypes (*Alicycliphilus*, *Agrobacterium*, and *Pseudoxanthomonas*) were identified in heterotrophic ECs. Two isolated autotrophic pure cultures, *Dechloromonas* sp. strain ECC1-pb1 and *Azospira* sp. strain ECC1-pb2, were able to grow by linking the oxidation of As(III) to As(V) with the reduction of ClO_3^-. The presence of the arsenite oxidase subunit A (*aroA*) gene was demonstrated with PCR in the ECs and pure cultures. This study demonstrates that ClO_3^- is an alternative electron acceptor to support the microbial oxidation of As(III).

The contamination of drinking water with arsenic (As) is a global public health issue. Arsenic is a human carcinogenic compound (2), which poses a risk to millions of people around the world (31). The most common oxidation states of As in aqueous environments are arsenite [As(III), H_3AsO_3] or arsenate [As(V), $H_2AsO_4^-$, and $HAsO_4^{2-}$]. Microbial processes play critical roles in controlling the fate and transformation of As in subsurface systems (22). As(V) binds to aluminum oxides more extensively than As(III) under circumneutral pH conditions (12, 16). Both As(III) and As(V) are strongly adsorbed on iron oxides (9). However, As(III) is more rapidly desorbed compared to As(V) (35).

Aerobic bacteria can oxidize As(III) forming As(V) (14, 28), which potentially is less mobile in the subsurface environment. Also, in environments with dissolved ferrous iron [Fe(II)] the oxidation of Fe(II) (both abiotic and biotic) would result in formation of Fe(III) (hydr)oxides such as ferrihydrite which adsorb As. Oxidation processes, therefore, can decrease the mobilization of As in groundwater. However, oxygen (O_2) is poorly soluble in groundwater and may become consumed by microbial activity, creating anaerobic zones. Alternative oxidants aside from O_2 also have the potential to support the microbial oxidation of As(III). Recently, several studies have demonstrated that nitrate-dependent As(III) oxidation is carried out by anaerobic microorganisms to gain energy from As(III) oxidation. As(III)-oxidizing denitrifying bacteria have been isolated from various environments including As-contaminated lakes and soil (21, 25), as well as enrichment cultures (ECs), and isolates from pristine sediments and sludge samples (33, 34). 16S rRNA gene clone library characterization of the ECs indicates that the predominant phylotypes were from the genus *Azoarcus* and the family *Comamonadaceae* (34).

Beside nitrate, chlorate (ClO_3^-) can also be considered as a possible alternative oxidant for microorganisms to promote the bioremediation of contaminated plumes (6, 17). (Per)chlorate is commonly used as a terminal electron acceptor by anaerobic bacteria; as a result, it is completely degraded to the benign end product, chloride (Cl^-). Microbial reduction of perchlorate proceeds via a three-step process of $ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow O_2 + Cl^-$ (6). Reduction of perchlorate to chlorate, and chlorate to chlorite is catalyzed by respiratory (per)chlorate reductases (3). Subsequent disproportionation of chlorite into Cl^- and O_2 is catalyzed by chlorite dismutase, which is the fastest step, and the O_2 produced is immediately consumed for energy of cell synthesis (6). Although organic compounds are the most well studied electron donors for (per)chlorate reduction, Fe(II) oxidation has also been shown to be linked to microbial ClO_3^- reduction (36).

The main objective of the present study is to explore the potential use of ClO_3^- as an electron acceptor for the microbial oxidation of As(III) by anaerobic bacteria. The theoretical stoichiometry of the reaction is presented below:

$$ClO_3^- + 3H_3AsO_3 \rightarrow Cl^- + 3HAsO_4^{2-} + 6H^+ \qquad (1)$$
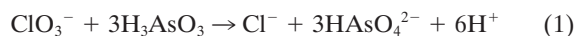
Based on bioenergetic considerations, the reaction is feasible as indicated by the highly exergonic standard change in Gibbs free energy [$\Delta G^{0'} = -92.4$ kJ mol^{-1} As(III)] calculated from $E^{0'}$ values of 0.618 and 0.139 V for ClO_3^-/Cl^- (6) and As(V)/As(III) (18), respectively.

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MATERIALS AND METHODS

Microorganisms. Sludge and sediment samples from different sources were used as inocula in the batch microcosms. Aerobic return activated sludge (RAS) and anaerobically digested sewage sludge (ADS) were obtained from a local municipal wastewater treatment plant (Ina Road, Tucson, AZ). Methanogenic granular sludge (biofilm pellets) samples were obtained from industrial upward-flow anaerobic sludge blanket bioreactors treating alcohol distillery wastewater (NGS; Nedalco, Bergen op Zoom, Netherlands). Chemolithotrophic As(III)-oxidizing denitrifying granular sludge was obtained from a 2-liter laboratory-scale As(III)-oxidizing denitrifying bioreactor biofilm (AODBBl; Department of Chemical and Environmental Engineering, University of Arizona). The total suspended solid (TSS) contents of the sludge and sediment samples were $6.32\% \pm 0.27\%$, $16.65\% \pm 0.21\%$, $6.08\% \pm 0.18\%$, and $1.63\% \pm 0.09\%$ wet weight basis, and the volatile suspended solids (VSS) contents were $5.96\% \pm 0.28\%$, $10.03\% \pm 0.10\%$, $5.76\% \pm 0.23\%$, and $1.12\% \pm 0.07\%$ wet weight basis for NGS, ADS, AODBB, and RAS, respectively. The inocula were stored under nitrogen gas at $4°C$.

Culture conditions. The standard basal medium was prepared by using ultrapure water (Milli-Q system; Millipore) and contained the compounds NH_4HCO_3 (3.16 mg liter^{-1}), $NaHCO_3$ (672 mg liter^{-1}), $CaCl_2$ (10 mg liter^{-1}), $MgSO_4 \cdot 7H_2O$ (40 mg liter^{-1}), K_2HPO_4 (300 mg liter^{-1}), $KH_2PO_4 \cdot 2H_2O$ (800 mg liter^{-1}), and 0.2 ml of a trace element solution liter^{-1} (described in the supplemental material). The final pH was 7.0 to 7.2.

Batch microcosms were performed in shaken flasks, which were incubated in a dark climate-controlled room at $30 \pm 2°C$. Serum flasks (160 ml) were supplied with 120 ml of a basal mineral medium containing bicarbonate as the only added carbon source. The medium was also supplemented with As(III) as electron donor and ClO_3^- as the electron acceptor. Details of the microcosm culture conditions are provided in the supplemental material.

Anaerobic ECs were established by adding ADS or RAS to the assays at 5% (vol/vol). The basal medium was amended with 0.5 mM As(III) ($NaAsO_2$) as the electron donor, 3.0 mM ClO_3^- ($NaClO_3$) as the electron acceptor, and 8 mM $NaHCO_3$ as the carbon source. Details of the enrichment process are described in the supplemental material. Autotrophic ECs were cultivated with $NaHCO_3$ as the sole added carbon source. Heterotrophic ECs were incubated with yeast extract (1.0 mg liter^{-1}) as an additional carbon source. Autotrophic ECs (ECC1 and ECC3), and heterotrophic ECs (ECC2 and ECC4) were originally derived from ADS and RAS, respectively.

Experimental incubations. Once the ECs had been established, they were used as inoculum for experiments testing growth and metabolism of As(III) under ClO_3^--reducing conditions. The experiments were monitored routinely by measuring As(III), As(V), ClO_3^-, and Cl^-. To avoid the contamination of ClO_3^- from ECs used as inoculum for fresh medium of the experiments, 5% volume of the EC was centrifuged in sterile tubes at $13,750 \times g$ for 10 min. The pellets were collected and washed with sterile Milli-Q water. The centrifugation and wash step was repeated. Finally, the pellets were resuspended into sterile Milli-Q water to a volume corresponding to 5% of the culture and transferred to inoculate the designed experiments. All assays were conducted in triplicate.

In two experiments, the impacts of low concentrations of organic carbon (OC) supplements were evaluated. In this case the basal medium was supplemented with yeast extract (YE; 1.0 mg liter^{-1}) or pyruvate (1.7 mg liter^{-1}). The experiments were inoculated with autotrophic and heterotrophic ECs, ECC1 and ECC2, at 1% (vol/vol).

In another experiment, the autotrophic and heterotrophic ECs (ECC1 and ECC2) were explored for their ability to utilize O_2 as an electron acceptor directly to oxidize As(III) to As(V) in the absence of ClO_3^-. In this case, the basal medium was supplemented with the initial amounts of total O_2 ranging from 0.25 to 25.0 mmol liter$_{liquid}^{-1}$, which achieved an equilibrium dissolved oxygen (DO) concentration of from 0.39 to 19.32 mg liter^{-1}, respectively.

16S rRNA gene clone libraries. Community genomic DNA was extracted from 5-ml samples taken from both autotrophic ECs (ECC1 and ECC3), and heterotrophic ECs (ECC2 and ECC4) by a modification of the extraction protocol described by the manufacturer for genomic DNA from bacteria (FastDNA spin kit for soil; Qbiogene, Inc., Carlsbad, CA). The procedure of 16S rRNA gene-based clone library analysis protocol and sequences used in the present study are described in detail by Sun et al. (34). Selected clones representing each phylotype obtained in each culture have been deposited in the GenBank database with following accession numbers: ECC1-1, GU056289; ECC2-1, GU056290; ECC3-2, GU056291; ECC1-2, GU056292; ECC2-2, GU056293; ECC3-3, GU056294; ECC1-7, GU056295; ECC2-18, GU056296; ECC3-1, GU056297; ECC4-8, GU056298; ECC2-7, GU056299; ECC2-23, GU056300; ECC3-6,

GU056301; ECC4-2, GU056302; ECC3-16, GU056303; ECC4-1, GU056304; and ECC4-3, GU056305.

To identify the isolates, the genomic DNA was extracted from washed pellets in a microcentrifuge tube resuspended in 1 ml of sterile Milli-Q water. DNA extraction and 16S rRNA gene sequencing of pure cultures were described by Sun et al. (34). Direct sequencing and cloning were conducted to confirm the purity of isolated culture based on the clean 16S rRNA gene DNA sequence from the extracted DNA of the intact culture, as well as verifying identical sequences of 24 sampled clones from 16S gene clone libraries performed on each isolate. The GenBank accession numbers for 16S rRNA genes of the pure cultures were GU202936 and GU202937 for *Dechloromonas* sp. strain ECC1-pb1 and *Azospira* sp. strain ECC1-pb2, respectively.

PCR primers for targeting the metabolizing genes. Two sets of specifically designed PCR primers were used to target the chlorite dismutase (*cld*) gene, including primer set DCD-F and DCD-R and primer set UCD-238F and UCD-646R (3). Two specifically designed PCR primer sets were used to identify two RuBisCO form genes, both *cbbL* and *cbbM* genes (10). Four primer sets were used to target the As(III) oxidase (*aroA*) gene (see Table S1 in the supplemental material) for all ECs and pure cultures, which included *aroA*1-85F and *aroA*1-593R (set 1) (14), *aroA*2-85F and *aroA*2-602R (set 2) (14), *aroA*3-69F and *aroA*3-1374R (set 3) (24), and *aroA*4-85F and *aroA*4-622R (set 4) (13). Two primer sets (see Table S1 in the supplemental material) were used to amplify the conserved regions of As(V) reductase genes (*arrA*), which included CH*arrA*fwd and CH*arrA*rev (set 1) (19) and HA*arrA*-D1F and HA*arrA*-G2R (set 2) (15). The PCR mixtures and cycling conditions were described in detail in each reference. To verify the integrity of the amplification, positive, negative (no template DNA), and original inoculum reactions were included. PCR results were checked on a 1.5% agarose gel. The identities of amplicons were also confirmed by verifying the molecular weight of the amplicons on the gel.

Analytical methods. As(III) and As(V) speciation was analyzed by high-performance liquid chromatography/inductively coupled plasma mass spectroscopy. Details of the method are provided in the supplemental material. ClO_3^-, ClO_2^-, Cl^-, and As(V) were analyzed by suppressed conductivity ion chromatography using a Dionex 500 system (Sunnyvale, CA) fitted with a Dionex IonPac AS11 analytical column (4 by 250 mm) and a AG16 guard column (4 by 40 mm). During each injection, the eluent (20 mM KOH) was used for 20 min.

Most-probable-number (MPN) assays were performed in sterile medium to count the cell number, which method is described in the supplemental material. Other analytical determinations (e.g., pH, TSS, VSS, etc.) were conducted according to standard methods (1).

RESULTS

Screening inoculum for anaerobic oxidation of As(III). All four inocula tested (RAS, ADS, NGS, and AODBB) displayed biological activity toward the anaerobic oxidation of As(III) with ClO_3^- as electron acceptor. Figure 1 illustrates the time course of As(III) (0.5 mM) conversion to As(V) for three of the inocula. In treatments incubated with ClO_3^- (3.0 mM), As(V) formation started after approximately 3 to 7 days and was concomitant with the disappearance of As(III). After approximately 27 days, the conversion was complete. The reactions were dependent on the presence of ClO_3^- and inoculum, as evidenced by the absence of any significant conversion in incubations lacking ClO_3^- or sludge inoculum (Fig. 1), or if the inoculum was heat killed (data not shown).

The average molar recovery of As(V) was 1.04 ± 0.11 mol As(V) formed per mol of As(III) consumed. Perchlorate was also used as an electron-acceptor to oxidize As(III) under anaerobic conditions with two (RAS and ADS) out of the four inocula tested (data not shown), but reaction times were longer, taking up to 48 days for the conversion to come to completion.

Enrichment cultures of As(III) oxidation linked to ClO_3^- reduction. MPN analysis of the As(III)-oxidizing, ClO_3^--respiring bacteria in the ECs were performed after the 13th and 23rd transfer of the ECs. The results (see Table S2 in the
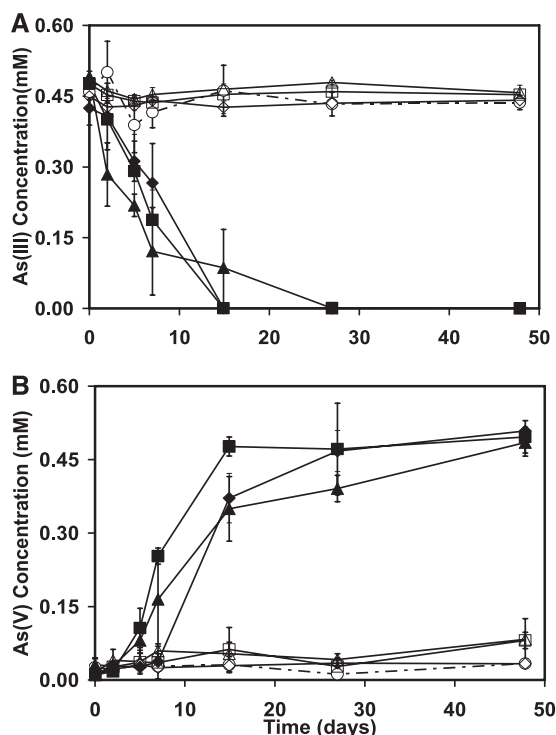
FIG. 1. (A and B) Removal of As(III) during the screening of pristine sludge inoculum (A) and formation of As(V) (B) by ADS, RAS, and NGS in the presence or absence of ClO_3^- under anaerobic conditions. Symbols: ADS (▲), RAS (■), and NGS (◆) with ClO_3^-; ADS (△), RAS (□), and NGS (◇) without ClO_3^-; abiotic (○). The symbols represent the means for triplicates in each treatment, and error bars indicate standard deviations.

supplemental material) indicate that 2.2×10^7, 2.4×10^7, 0.33×10^7, and 1.7×10^7 cells were present per ml of ECC1, ECC2, ECC3, and ECC4, respectively, after the 23rd transfer; corresponding to 6.5×10^{12} to 4.4×10^{13} cells produced per mol of As(V) formed.

ECC1 and ECC2 derived from ADS were tested for their ability to utilize inorganic and organic carbon sources for growth under anaerobic conditions. The autotrophic ECC1 and heterotrophic ECC2 were incubated with As(III) (0.9 mM) both in the presence and in the absence of ClO_3^- (1.5 mM). The time course from these incubations is provided in Fig. 2 (panels A and B [ECC1] and panels C and D [ECC2]), respectively. In ECC1, As(V) formation came to completion after 28 to 30 days both in the presence and in the absence of YE (1 mg liter^{-1}). The maximum reaction rates were 0.13 ± 0.03 and 0.20 ± 0.08 mM As(V) formed per day for the treatments with and without YE, respectively. In ECC2, it took only 12 to 15 days to complete the oxidation of As(III) to As(V) when YE or pyruvate (1.7 mg liter^{-1}) was added. In contrast to ECC1, ECC2 did not convert As(III) to As(V) without an OC addition, indicating a strict requirement for OC. The maximum rate was 0.34 ± 0.08 and 0.25 ± 0.02 mM As(V) formed per day for the treatments with YE and pyruvate additions, respectively. Both were higher than those for ECC1.

The possible products of ClO_3^- respiration, including ClO_2^- and Cl^-, were also monitored to determine the end products of ClO_3^- reduction. Figure 3 demonstrates that Cl^-

was the only end product in the treatment inoculated with ECC1 (Cl^- corresponded to 98.1% ± 1.8% of the measured removal of ClO_3^-). There was no ClO_2^- observed during the experiment (data not shown). The molar ratios of As(III) consumption or As(V) formation to the net ClO_3^- consumption (corrected for endogenous ClO_3^- consumption) or net Cl^- formation (corrected for endogenous Cl^- formation) are provided in Table S3 in the supplemental material. The molar ratios of As(V) formation to ClO_3^- consumption or Cl^- formation were 2.91 ± 0.48 and 2.98 ± 0.05, respectively, which were close to the theoretical ratio of 3.0 expected for As(III) oxidation linked to complete ClO_3^- reduction to Cl^- from equation 1 above. The ECC2 also coupled the oxidation of As(III) to the reduction of ClO_3^- (at molar ratios approaching 3), forming Cl^- as the sole end product (see Table S3 in the supplemental material).

Since O_2 is formed as an intermediate during the reduction of ClO_3^-, an experiment was conducted to evaluate whether both autotrophic and heterotrophic ECs could utilize O_2 directly as an electron acceptor to oxidize As(III) to As(V) in the absence of ClO_3^-. An example of the time course from these incubations is provided in Fig. S1 in the supplemental material for ECC1 cultured under autotrophic conditions. The results showed that ECC1 has the ability to effectively oxidize As(III) to As(V) only in the presence of low O_2 concentrations. Reactions were only evident if the initial O_2 was supplied at 5.0 mmol liter$_{liquid}^{-1}$ (DO at 6.53 mg liter^{-1}) or less. The reaction was completely inhibited when O_2 was supplied at 12.5 and 25 mmol liter$_{liquid}^{-1}$ (DO at 12.97 and 19.32 mg liter^{-1}). The O_2-dependent As(III) oxidation activity of ECC1 ranged from 0.17 to 0.58 mM As(V) formed per day, with the highest activity of 0.58 mM As(V) formed per day at a optimum initial O_2 concentration of 2.5 mmol liter$_{liquid}^{-1}$ (DO at 3.57 mg liter^{-1}) (see Fig. S1C in the supplemental material). No oxidation of As(III) occurred in the abiotic control with the same O_2 concentration.

Microbial community composition of ECs. Rarefaction analysis (see Fig. S2 in the supplemental material) suggested that 24, 32, 42, and 32 clones were sufficient for capturing the community composition in the clone libraries corresponding to ECC1, ECC2, ECC3, and ECC4, respectively. A total of nine unique phylotypes were obtained from the four cultures. The 130 clones analyzed in the present study fell into three phylogenetic divisions, *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*, accounting for 2.4, 82.6, and 15.0% of all of the clones from the four cultures, respectively. The phylotypes recovered from each EC are shown in Fig. S3 in the supplemental material. The *Betaproteobacteria* accounted for a large majority of all of the clones and six of the nine of the phylotypes recovered were most closely related to members of this group (*Dechloromonas*, *Azospira*, *Azonexus*, *Achromobacter*, *Acidovorax*, and *Alicycliphilus*). One phylotype belonged to the *Alphaproteobacteria* and was most closely related to *Agrobacterium*. Two phylotypes belonged to the *Gammaproteobacteria* and were most closely related to *Stenotrophomonas* and *Pseudoxanthomonas*.

Four of the nine phylotypes identified in the ECs dominated in terms of the number of clones recovered. One of these phylotypes had 99% sequence similarity to the genus *Dechloromonas* according to the RDP Classifier (8). The *Dechloromo-*
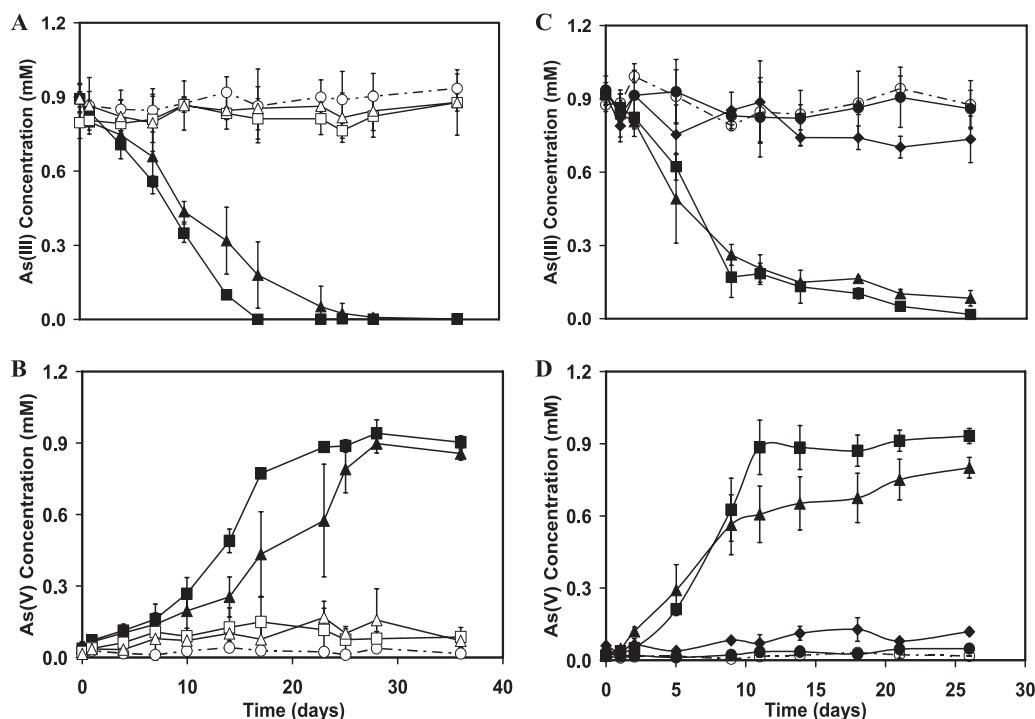
FIG. 2. (A and B) Removal of As(III) (0.9 mM) (A) and formation of As(V) (B) by ECC1 in the presence of ClO_3^- (1.5 mM) under anaerobic conditions. Symbols: full treatment with As(III) + ClO_3^-, without YE (▲) and with YE (■); live controls with only As(III) added included As(III) without YE (△) and with YE (□); abiotic control with As(III) and ClO_3^- (○). (C and D) Removal of As(III) (0.9 mM) (C) and formation of As(V) (D) by ECC2 in the presence of ClO_3^- (1.5 mM) under anaerobic conditions. Symbols: full treatment with As(III) + ClO_3^-, without OC (◆), with YE (■), and with pyruvate (▲); control with only As(III) added without OC (●); abiotic control with As(III) and ClO_3^- (○). The symbols represent the means for triplicates in each treatment, and error bars indicate standard deviations.

nas phylotype accounted for 29.2 to 56.3% of the clones recovered in the clone libraries prepared from ECC1, ECC2 and ECC3. The other two dominant phylotypes were related to the genera *Azospira* (99% sequence similarity) or *Azonexus* (97% sequence similarity). The three above-mentioned phylotypes belonged to the family *Rhodocyclaceae*, and at least one or more of them were present as a dominant phylotype in the clone libraries from each of the four ECs. The fourth dominant phylotype was related to the genera *Stenotrophomonas* (97% sequence similarity), and it accounted for 6.3 to 20.8% of the clones in the libraries prepared from each EC.

The remaining five phylotypes accounted for small fractions of the clones examined. One phylotype was closely related to *Achromobacter* (99% sequence similarity) and occurred in ECC3 and ECC4. Phylotypes closely related to *Acidovorax* (100% sequence similarity) and *Pseudoxanthomonas* (100% sequence similarity) were present in ECC3 and ECC4, respectively. Two other phylotypes closely related to *Alicycliphilus* (98% sequence similarity), and *Agrobacterium* (100% sequence similarity) were present in ECC2.

Anaerobic oxidation of As(III) by pure cultures. Two dominant phylotypes were isolated by dilution to extinction using the MPN method. *Dechloromonas* sp. strain ECC1-pb1 and *Azospira* sp. strain ECC1-pb2 were isolated from ECC1. The time course of the ClO_3^--dependent As(III) oxidation by the two pure cultures are shown in Fig. 4. Treatments with live cells of *Dechloromonas* sp. strain ECC1-pb1 and *Azospira* sp. strain ECC1-pb2 converted As(III) to 0.52 ± 0.03 mM and

0.54 ± 0.02 mM As(V), respectively, after 6 days. Controls lacking cells or with heat-killed cells of the isolates converted As(III) at less than 0.02 mM (data not shown). Likewise, there was less than 0.03 mM conversion of the As(III) in controls with live cells but lacking ClO_3^- addition. The cell numbers based on the MPNs of the two isolated pure cultures were also shown to increase with As(III) oxidation, which illustrated the capacity of each culture to gain energy from the reaction of As(III) oxidation coupled to ClO_3^- reduction (Fig. 4).

PCR. PCR targeting *cld*, RuBisCO, *aroA*, and *arrA* genes were performed on all four ECs and the two pure cultures. The electrophoresis gels (data not shown) showed that *cld* were present in all of the As(III)-oxidizing, ClO_3^--reducing cultures. RuBisCO genes were detected in ECC1, ECC3 and the two pure cultures. Various primer sets were used to detect the existence of *aroA* or *arrA* genes. However, only *aroA* genes of primer set 4 were successfully detected, and they were detected in all four ECs and the two pure cultures.

DISCUSSION

Evidence of biological oxidation of As(III) to As(V) linked to chlorate reduction. In the present study, the capacity of microorganisms from diverse anaerobic samples to utilize ClO_3^- as an electron acceptor for the oxidation of As(III) was demonstrated. The biological nature of the reaction is inferred from the lack of any conversion in uninoculated samples and heat-killed samples. The detection of As(III) oxidase (*aroA*)
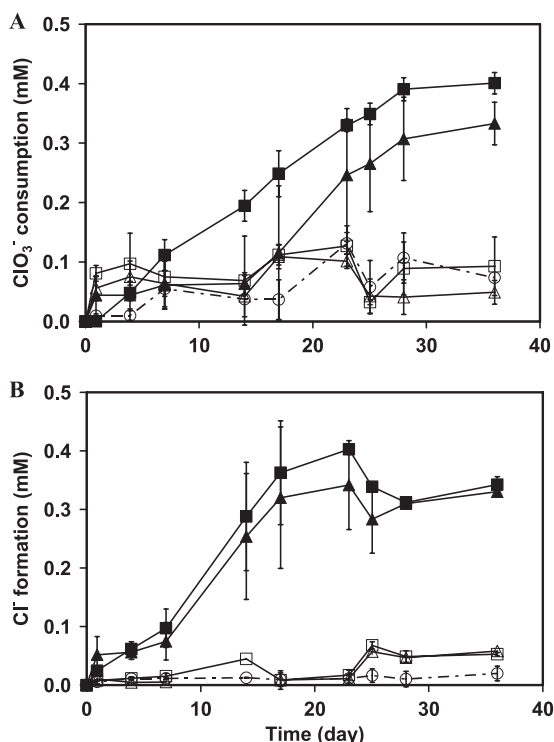
FIG. 3. ClO_3^- reduction at a initial aqueous As(III) concentration (0.9 mM). The consumption of ClO_3^- (1.5 mM) (A) and the formation of Cl^- (B) by ECC1 under anaerobic conditions is depicted. Symbols: full treatment with As(III) and ClO_3^-, without YE (▲) and with YE (■); endogenous control with only ClO_3^- added, without YE (△), and with YE (□); abiotic control with As(III) and ClO_3^- (○). The symbols represent the means for triplicates in each treatment, and error bars indicate standard deviations.
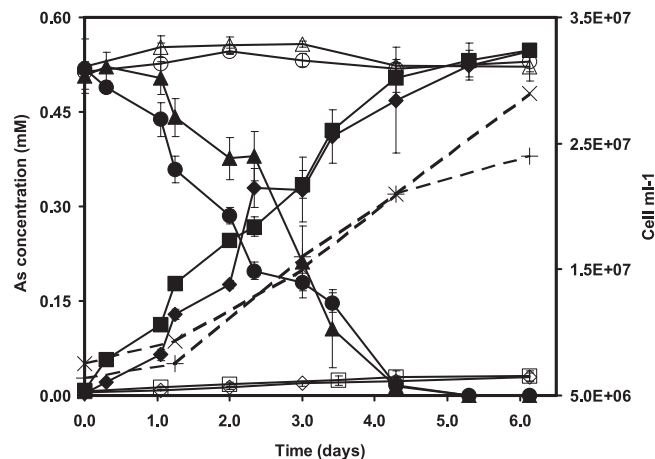

FIG. 4. As(III) oxidation (0.5 mM) linked to ClO_3^- reduction by two isolated pure cultures, *Dechloromonas* sp. strain ECC1-pb1 and *Azospira* sp. strain ECC1-pb2. Symbols for *Dechloromonas* sp. strain ECC1-pb1 in the treatment with As(III) and ClO_3^-: As(III) removal (▲), As(V) formation (◆), and cell growth (×). Symbols for *Dechloromonas* sp. strain ECC1-pb1 in the treatment with As(III) without ClO_3^-: As(III) removal (△) and As(V) formation (◇). Symbols for *Azospira* sp. strain ECC1-pb2 in the treatment with As(III) and ClO_3^-: As(III) removal (●), As(V) formation (■), and cell growth (+). Symbols for *Azospira* sp. strain ECC1-pb2 in the treatment with As(III) without ClO_3^-: As(III) removal (○) and As(V) formation (□). The symbols represent the means for triplicates in each treatment, and error bars indicate standard deviations.

genes in ECs and isolates provides molecular evidence for the biological As(III) oxidation capacity. Both autotrophic and heterotrophic ECs linked the oxidation of As(III) to ClO_3^- reduction. The bacteria gain energy from the process, as evidenced by the ECs being sustained after a large number of transfers (23 total). Moreover, growth of the two isolated pure cultures confirm the bacteria gain useful energy when they couple As(III) oxidation to ClO_3^- reduction.

The biological conversion of As(III) to As(V) in the absence of exogenous O_2 was dependent on the presence of ClO_3^-. No As(III) oxidation occurred in the inoculated incubations lacking ClO_3^-. The concentration of As(V) formed in the presence of ClO_3^- corresponded to the concentration of As(III) removed, indicating that As(V) was the main product of the conversion and that there was no significant formation of other As metabolites. The presence of As(III) greatly enhanced ClO_3^- consumption beyond the background endogenous consumption, suggesting its role as the primary electron donor for the microbial reaction. The production of Cl^- in the incubations was linked to the addition of As(III), and the yield of Cl^- corresponded to the expected stoichiometry of 3 based on the electron equivalents in As(III). If the As(III) oxidation was linked only to the O_2 generated from ClO_3^- reduction, the ratio of As(III) removed to ClO_3^- consumption would have been approximately 2. Thus, these results confirm that oxida-

tion of As(III) to As(V) was successfully linked to the complete ClO_3^- reduction process, including both the initial reduction of ClO_3^- to ClO_2^- and the reduction of O_2 formed from the dismutation of ClO_2^- to Cl^-. Evidence for the latter is based on having demonstrated that the autotrophic EC can utilize low concentrations of O_2 as an electron acceptor directly to oxidize As(III) and PCR detection of *cld* (3), the gene coding for ClO_2^- dismutase that generates O_2.

The present study adds ClO_3^- to the pool of known electron acceptors that can be used by microorganisms to oxidize As(III) in anaerobic environments. Previously, anoxic oxidation of As(III) was demonstrated when NO_3^- was used as an electron acceptor with bacterial cultures isolated from As contaminated (21, 25) or pristine sites (34). Likewise, selenate-dependent-As(III)-oxidizing bacteria have been described (11).

The As(III)-oxidizing bacterial community: *Rhodocyclaceae* and *Stenotrophomonas*. Four phylotypes of clones were dominant from the 16S rRNA gene clone libraries. All ECs examined contained clones representing phylotypes within the family *Rhodocyclaceae* (Fig. 5). One of the *Rhodocyclaceae* phylotypes recovered from three ECs was *Dechloromonas*, which is 99% similar to *Dechloromonas* sp. strain JM (20) and *Dechloromonas* sp. strain HZ (38), known to utilize hydrogen to reduce perchlorate. Strain HZ is also known to grow autotrophically (38).

A second *Rhodocyclaceae* phylotype closely related to the genus *Azospira* was also obtained from three of the ECs. This phylotype has 99% sequence similarities with *Dechlorosoma suillum* PS (syn. *Azospira oryzae* PS) and *Dechlorosoma* sp. strain Iso1, known for their ability to reduce (per)chlorate (7).
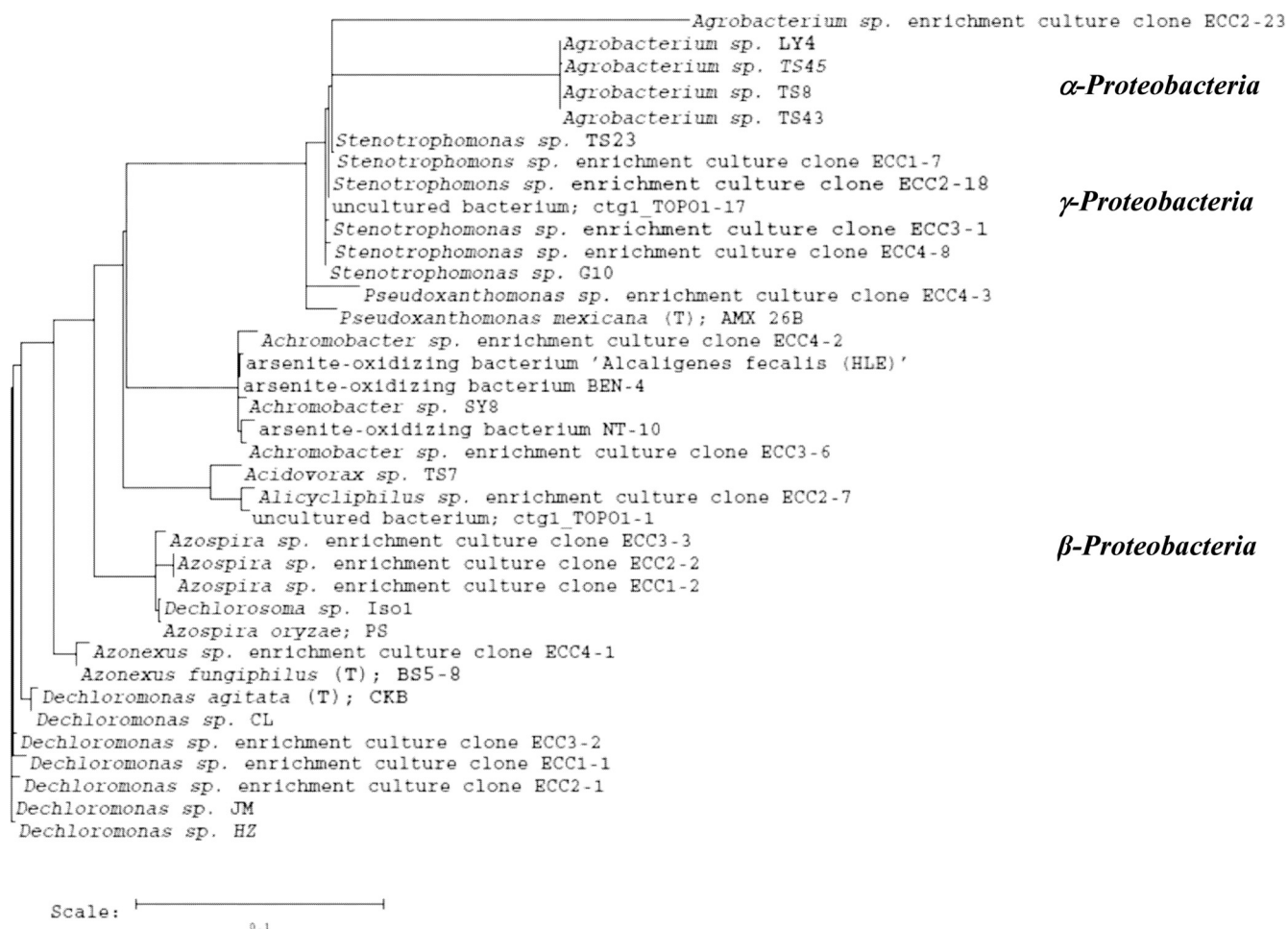
FIG. 5. Phylogenetic relationships of the EC clones recovered in the present study. Maximum-likelihood analysis was used to generate the tree from 16S rRNA gene sequences of bacterial strains and unique phylotypes represented by clones recovered from the four cultures labeled with "ECC#-#" (the first number refers to the EC, the second number refers to the clone number).

A third *Rhodocyclaceae* phylotype closely related to the genus *Azonexus* (Fig. 5) was found in ECC4, and it has 98% sequence similarity to *Azonexus fungiphilus* strain BS5-8 (23). Strains in the *Rhodocyclaceae* are also known for their ability to link Fe(II) oxidation to (per)chlorate reduction (36). These include *Dechloromonas agitata* strain CKB with 98% sequence similarity to the *Dechloromonas* clones and *Azospira oryzae* PS with 99% sequence similarity to several *Azospira* clones.

The fourth dominant phylotype was related to the genus *Stenotrophomonas*, and it was present in all of the ECs studied. The *Stenotrophomonas* phylotype was most closely related to an uncultured bacterium clone EC1-17 (99%) from an As(III)-oxidizing denitrifying EC (34). The phylotype was also closely related (98% sequence similarity) to aerobic isolates of *Stenotrophomonas* spp. T23 (5) known for their ability to oxidize the As(III) to As(V).

The As(III)-oxidizing bacterial community: less dominant members. Less dominant phylotypes were those accounting for less than 16% of all clones in a given clone library. Among these was a phylotype with 99% similarity to aerobic heterotrophic As(III)-oxidizing bacteria *Alcaligenes faecalis* (HLE) and *Achromobacter* sp. strain NT10 isolated from gold mine

sites in Australia (28). All of these isolates were heterotrophic As(III)-oxidizing bacteria. Two of the minor phylotypes are related to a cluster of genera (*Acidovorax* and *Alicycliphilus*) within the family *Comamonadaceae* (Fig. 5). One of these phylotypes has 99% sequence similarity to *Acidovorax* sp. strain TS7, which was isolated from highly As-contaminated soils (5) (Fig. 5). The other was related to the genus *Alicycliphilus* and an uncultured bacterium clone EC1-1, which was recovered from an anaerobic chemolithotrophic As(III)-oxidizing denitrifying EC (34). Another minor phylotype has 97% sequence similarity to aerobic heterotrophic As(III)-oxidizing bacteria *Agrobacterium* sp. strains TS43, TS45, and LY4 isolated from soil samples with various levels of arsenic contamination (5) (Fig. 5).

Characterization of anaerobic As(III)-oxidizing chlorate-respiring bacteria. The As(III) oxidizers in the autotrophic ECs and pure cultures are chemolithotrophic, obtaining energy from oxidation of As(III). ECC1, ECC3, and two isolates, which received no OC supplements, most likely obtained carbon from HCO_3^-/CO_2 added to the medium and headspace. After 23 transfers without addition of any OC there would not be any reason to expect residual endogenous OC remaining in

the ECs, indicating these ECs were most likely autotrophic. This was further supported by the detection of RuBisCO genes. Two of the ECs (ECC2 and ECC4) were maintained with low levels (1 mg liter^{-1}) of YE supplements. These cultures were obligate heterotrophic, as evidenced by the fact that ECC2 and ECC4 had no activity in the incubations without addition of OC and by the fact they had no detectable RuBisCO genes.

O_2 is produced as an intermediate during ClO_3^- reduction. However, this O_2 is rapidly consumed for cell respiration so that it does not accumulate in solution to high levels (29, 32). Previous studies have illustrated that biological oxidation of As(III) by aerobic bacteria is very fast and efficient in the presence of O_2 (27). Our results showed that when low O_2 concentrations were supplied as the sole electron acceptor, As(III)-oxidizing, ClO_3^--respiring bacteria from autotrophic ECC1 could utilize O_2 to oxidize As(III) to As(V). In contrast, exposure to higher O_2 levels caused inhibition of the activity, indicating ClO_3^--reducing microorganisms in the ECs are microaerophilic. Most of the known dissimilatory (per)chlorate-reducing bacteria are facultative anaerobic or microaerophilic (7, 26). For example, two hydrogen-utilizing perchlorate-reducing bacteria, *Dechloromonas* sp. strains JDS5 and JDS6, are microaerophilic and incapable of growth at atmospheric O_2 concentrations (30). It is well known that high concentrations of DO inhibit the activity of (per)chlorate-reducing bacteria (32). ClO_3^- reductase is sensitive to O_2 exposure (37). Although chlorite dismutase activity is not inhibited by O_2, expression of this enzyme is not observed in ClO_3^--reducing bacteria under aerobic conditions even in the presence of ClO_3^- (4).

ACKNOWLEDGMENTS

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